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Statins activate a mitochondria-operated pathway of apoptosis in breast tumor cells by a mechanism regulated by ErbB2 and dependent on the prenylation of proteins

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Abstract **Statins are inhibitors of the mevalonate synthesis pathway that induce apoptosis in tumor cells although the apoptotic mechanism activated by statins remains to be elucidated. We have examined the role of the mitochondria-operated pathway of apoptosis in the cell death induced by statins in breast tumor cells and its regulation by protein prenylation and ErbB2 overexpression. Lovastatin treatment down-regulates the expression of Bcl-2 and activates apoptosis through a mitochondria-operated, ErbB2- regulated mechanism. Apoptosis induced by statins is independent of their effects on cholesterol synthesis and involves protein prenylation. Our results indicate that prenylation of apoptosis-regulating proteins is a key event in the survival of breast tumor cells and this requirement could be circumvented in cells overexpressing the oncogene ErbB2.**

Keywords: Statin; Apoptosis; Bcl-2; ErbB2; Mitochondria; Prenylation

1. Introduction

Blocking of mevalonate synthesis by statins, inhibitors of the 3-OH-3-methyl-glutaryl CoA (HMG-CoA) reductase, has been used to treat hypercholesterolemia and prevent cardiovascular diseases [1,2]. However, statins not only reduce cholesterol levels but they also affect the synthesis of other products of the mevalonate pathway such as isoprenoids, essential components of signalling proteins involved in cell proliferation and cancer [3–5]. Attachment of isoprenoids compounds to protein members of the Ras/Rho family facilitate their anchoring to the cell membrane where they carried out their role [6].

Clinical studies have reached mixed conclusions about the relationship between statin use and breast cancer risk [7]. On the other hand, statins have been shown to inhibit cell proliferation and induce cell death by apoptosis in different tumor cell lines [8–10]. Statins seem to induce apoptosis and inhibit proliferation to a greater degree in malignant than in non-malignant cells, possibly because of the increased expression of HMG-CoA reductase and a greater requirement for mevalonate-derived isoprenoids in tumour as opposed to normal cells [11]. In breast tumor cells, different signalling pathways have been reported to be responsible for the apoptosis induced by statins. Nitric oxide and lipid rafts levels and JNK activation have been implicated in the proapoptotic actions of statins [9,12]. Down-regulation of survivin and inhibition of Akt have also been observed in statin-induced apoptosis in other tumor cells [13,14]. However, the apoptotic mechanism activated in breast tumor cells by statins remains to be elucidated.

The *ErbB2* proto-oncogene encodes human epidermal growth factor receptor-2 which belongs to the epidermal growth factor receptor tyrosine kinases. Upregulation of erbB2 receptors has been found in about 25–30% breast cancer patients, which is related with poor prognosis. ErbB2 overexpression is linked to resistance to apoptosis and down-regulation of erbB-2 receptor by trastuzumab (herceptin) enhances death receptor-induced apoptosis [15]. In this study we show that statin treatment down-regulate Bcl-2 expression and induces apoptosis in breast tumor cells by a mitochondria-operated pathway. In breast tumor cells overexpressing ErbB2 apoptosis by statin is clearly inhibited. Apoptosis induced by statins in breast tumor cells is independent of their effects on cholesterol synthesis and requires the prenylation of proteins.

2. Materials and methods

2.1. Reagents and antibodies

Caspase inhibitor benzyloxy-carbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (Z-VAD-FMK) was from Bachem, AG (Bachem, Bubendorf, Switzerland). Mevinolin (lovastatin), squalene, mevalonic acid, mevastatin, geranylgeranylpyrophsphate (GGPP), farnesylpyrophsphate ammonium salt (FPP) and anti-tubulin antibody were purchased from Sigma Chemical Corp (St. Louis, MO). Anti-human caspase-8 monoclonal antibody was purchased from Cell Diagnostica (Münster, Germany). Anti-poly(ADP-ribose) polymerase (PARP), cytochrome *c* and Bax monoclonal antibodies were from Biosciences PharMingen (San José, CA). Anti-caspase-9 and caspase-3 antibodies were from New England Biolabs Inc. (Ipswich, MA). Anti-GAPDH antibody was from Biogenesis (Poole, UK). Anti-ErbB2 antibody was kindly provided by Dr. Joaquin Arribas (Vall d'Hebron University Hospital Research Institute, Barcelona, Spain). Anti-Bcl-2 and secondary goat anti-mouse and goat anti-rabbit antibodies were obtained from DAKO (Cambridge, United Kingdom).

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Abbreviations: FPP, farnesylpyrophosphate ammonium salt; GGPP, geranylgeranylpyrophosphate; PARP, poly-(ADP-ribose) polymerase; HMG-CoA, hydroxymethylglutaryl-Coenzyme A

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2.2. Cell lines

Stable MCF-7 cell line overexpressing human Bcl-2 protein has been described previously [16]. MCF-7 cell line overexpressing ErbB2 was a donation of Dr. Joaquin Arribas (Vall d'Hebron University Hospital Research Institute, Barcelona, Spain). The cell lines were either maintained in RPMI 1640 (MDA-MB231, MCF-7) or in DMEM (MDA-MB435S, SkBr3 and BT474) containing 10% fetal bovine serum and 1 mM L-glutamine at 37 °C in humidified 5% CO2, 95% air incubator. Culture medium of MDA-MB435S cells (DMEM) was also supplemented with insulin (10 μg/mL). MCF10A cells were maintained in DMEM/F12 (1:1) containing 10% fetal bovine serum, insulin (10 Hg/mL), EGF (20 ng/mL) and hydrocortisone (500 ng/mL).

2.3. Analysis of apoptosis

Cells (3×10^5/well) were treated with statin in 6-well plates for the times indicated in the figure legends. After treatment, hypodiploid apoptotic cells were detected by flow cytometry according to published procedures [16]. Basically, cells were washed with phosphate buffered saline (PBS), fixed in cold 70% ethanol and then stained with propidium iodide while treating with RNAse. Quantitative analysis of subG1 cells was carried out in a FACSCalibur cytometer using the Cell Quest software (Becton Dickinson, Mountain View, CA). Phosphatidylserine (PS) exposure on the surface of apoptotic cells was examined by flow cytometry after staining with Annexin-V-FLUOS (Roche Molecular Biochemicals, Germany), following instructions provided by the manufacturer.

2.4. Viability assay

Cells (3.5×10^3/well) were treated with statin in 24-well plates for the times indicated in the figure legends. After treatment, cells were washed with PBS and stained with 0.2% Crystal Violet in 2% Ethanol for 20 min at room temperature. Absorbance of the samples was determined using a multiplate reader Varioskan Flask (Termo Electron Corporation).

2.5. Cellular fractionation

Cells (3×10^5/well) were treated with statin in 6-well plates for the times indicated in the figure legends. After treatment, cells were detached from the plate with RPMI/EDTA and trypsin, washed with PBS and lysed in 30 μl ice cold lysis buffer (80 mM KCl, 250 mM sucrose, 500 μg/ml digitonin and protease inhibitors in PBS). For measurements of cytochrome *c* and Bax, cell lysates were centrifuged for 5 min at $10000 \times g$ to separate the supernatant (cytosolic fraction) and pellet (mitochondria-containing fraction). Amount of protein in each fraction was determined by the Bradford protein assay (Bio-Rad, Hertfordshire, UK). Proteins from the supernatant and pellet were mixed with Laemmli buffer and resolved on SDS–12% PAGE minigels. Cytochrome *c* and Bax were determined by Western blot analysis.

2.6. Detection of proteins by Western blot analysis

Cells (3×10^5/well) were treated with statin in 6-well plates for the times indicated in the figure legends. After treatment, cells were detached from the plate with RPMI/EDTA and trypsin, washed with PBS and lysed in 20 μl of Laemmli buffer. Samples were sonicated and proteins were resolved on SDS–polyacrylamide minigels and detected as described previously [17].

2.7. Statistical analysis

All data are presented as the means ± S.E. of at least three independent experiments. The differences among different groups were determined by the Student's *t*-test. $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Lovastatin induces apoptosis in human breast tumor cells

Antitumor properties of statins have been extensively evaluated [7]. Despite these studies, there are still important controversies regarding the beneficial effects of statins on breast tumor prevention. On the other hand, antiproliferative and proapoptotic actions of statins on breast tumor cells have been recently examined [9,18,19]. However, the molecular mechanism by which statins induce apoptosis in breast tumor cells remains unknown. In this report, we have examined the effect of the lypophilic statin lovastatin on the mitochondrial pathway of apoptosis in breast tumor cells. Lovastatin-induced apoptosis in a dose and time-dependent manner in the breast tumor cell line MDA-MB231 as determined by measuring the percentage of hypodiploid cells (Fig. 1A). Activation of apoptosis by lovastatin was also observed in the highly metastatic breast tumor cell line MDA-MB435S (Fig. 1B). In contrast, at the doses used in these experiments the ErbB2-overexpressing breast tumor cell lines BT474 and SKBr-3 were very resistant to lovastatin-induced apoptosis (Fig. 1B). To further investigate the importance of ErbB2 overexpression on the resistance of breast tumor cells to statin-induced apoptosis, we performed a number of experiments with MCF-7 cells that has been transfected with a cDNA encoding for the oncogene ErbB2. These cells stably overexpress ErbB2 as can be seen in Fig. 1C. Next, we examined the effect of lovastatin treatment in control and ErbB2-overexpressing MCF-7 cells. Results shown in Fig. 1C demonstrate that MCF-7/ErbB2 cells are markedly resistant to lovastatin treatment as determined by analysis of phosphatidylserine exposure on the surface of apoptotic cells after staining with Annexin-V-FLUOS and cell viability with crystal violet. Interestingly, the immortalised human breast epithelial cell line MCF10A was also markedly resistant to the proapoptotic effect of lovastatin (not shown).

3.2. Lovastatin induces the activation of a caspase-dependent, mitochondria-operated pathway of apoptosis in breast tumor cells

To further establish the mechanism of lovastatin-promoted cell death, we examined the caspase dependency of this cell death process. We found that the generation of subG1 cells induced by lovastatin was dependent on caspase activation as it was completely prevented by the general caspase inhibitor Z-VAD-fmk (Fig. 2A). To confirm that the apoptosis cascade was fully active in MDA-MB231 cells treated with lovastatin and that caspase activation was involved in the process, we analyzed the proteolytic degradation of the nuclear protein PARP, a substrate of executioner caspases. As shown in Fig. 2B, PARP cleavage was clearly induced in cells treated with lovastatin. Moreover, we observed that the initiator caspases in the extrinsic and intrinsic pathways of apoptosis (caspase-8 and caspase-9) were activated by lovastatin in MDA-MB231 (Fig. 2C) and MCF-7 cells (not shown). After a 48 h-treatment with lovastatin, caspase-3, an executioner caspase, was also activated in the MDA-MB231 cell line (Fig. 2C).

To examine the mechanism of lovastatin-induced apoptosis we first analysed the activation of a mitochondria-controlled apoptotic pathway by lovastatin in breast tumor cells. To this end we determined the translocation of cytosolic Bax to mitochondria and the release of cytochrome *c* from this organelle. Analysis by Western blotting of cytosolic and mitochondria-containing subcellular fractions revealed that treatment of breast tumor MCF-7 cells with lovastatin induced the loss of cytochrome *c* from the mitochondrial fraction and the translocation of Bax from the cytosol to the mitochondria-containing fraction (Fig. 3A). Interestingly, in MCF-7 cells overexpressing

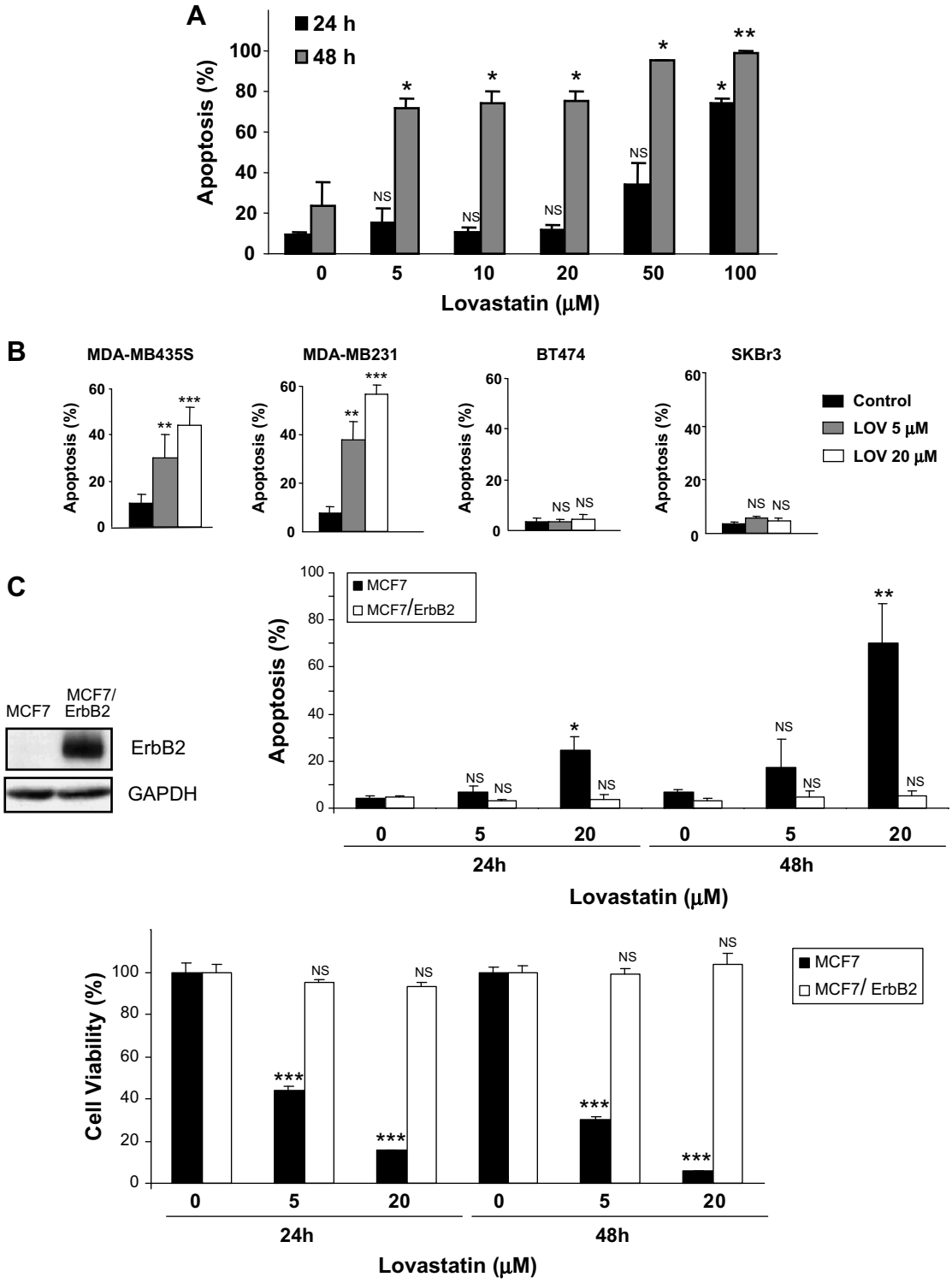

Fig. 1. Lovastatin induces apoptosis in breast tumor cells. (A) MDA-MB231 cells were seeded in 6-well plates (3×10^5 cells/well) and treated with lovastatin as indicated either for 24 h or for 48 h in medium containing 0.5% foetal bovine serum. After this incubation apoptosis was assessed by measuring the percentage of hypodiploid cells in the culture. Results show the means ± S.E. of three independent experiments. (B) MDA-MB435s, MDA-MB231, BT474 and SBr3 cells were seeded in 6-well plates (3×10^5 cells/well) and treated with lovastatin (5 or 20 μM) in medium containing 0.5% foetal bovine serum. The percentage of apoptotic cells with sub-G1 DNA content are shown and the values represent means ± S.E. of three independent experiments. (C) MCF7 control and MCF7-overexpressing ErbB2 cells were seeded in 6-well plates (3×10^5 cells/well, Annexin-V-Fluos) or 24-well plates (3.5×10^3/well, crystal violet) and treated with lovastatin (5 or 20 μM) in medium containing 0.5% foetal bovine serum. The percentage of cells with externalization of phosphatidylserine (Annexin-V-Fluos) or cell viability (crystal violet) are shown and the values represent means ± S.E. of four independent experiments. Immunoblot detection of ErbB2 expression is also shown.

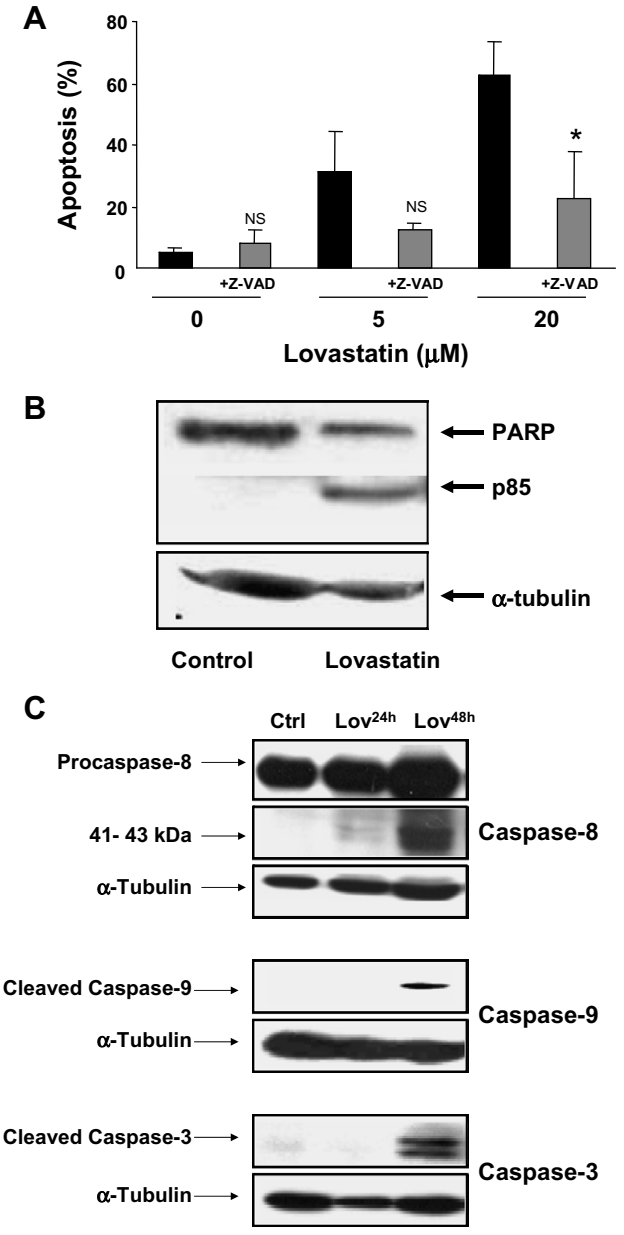

Fig. 2. Caspase-dependent activation of a mitochondria-operated pathway of apoptosis by lovastatin in breast tumor cells. (A) MDA-MB231 cells were seeded in 6-well plates (3×10^5 cells/well) and incubated with or without Z-VAD-fmk (100 μM) 1 h before the addition of lovastatin. After 48 h, apoptosis was determined as indicated in Fig. 1A. Results show the means ± S.E. of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ as compared to cells without z-VAD-fmk (B) Total lysates from MDA-MB231 cells (3×10^5) treated with lovastatin (100 μM) for 48 h in the presence of 10% serum were analyzed by immunoblotting for PARP cleavage. α-Tubulin levels were used as a loading control. Similar results were obtained in two independent experiments. (C) MDA-MB231 cells were seeded in 6-well plates (3×10^5/well) and treated with lovastatin (100 μM in the presence of 10% serum) for either 24 h or 48 h. Total protein was harvested and caspase processing was determined by Western blot analysis. Similar results were obtained in two independent experiments.

Bcl-2 [16] both events were clearly inhibited (Fig. 3A). To get further insight into the mechanism of lovastatin-induced apoptosis we studied the expression levels of Bcl-2 in breast tumor cells treated with this statin. Western blots revealed that at 24 h and 48 h after the addition of lovastatin there was a decrease in the expression of Bcl-2 protein in MCF-7 and MDA-MB231 cells (Fig. 3B). These data suggest that inhibiting HMG-CoA reductase activity in breast tumor cells caused the down-regulation of antiapoptotic Bcl-2 protein that in turn promoted the activation of a mitochondria-operated pathway of apoptosis.

3.3. Statin-induced apoptosis requires geranylgeranylation and farnesylation of proteins but not the synthesis of cholesterol

We next studied the relevance of the inhibition by lovastatin of mevalonate synthesis, the immediate downstream product of HMG-CoA reductase, on apoptosis. Interestingly, the apoptosis-inducing effects of lovastatin in both MDA-MB231 and MDA-MB435S cells were prevented by the presence of mevalonate in the culture medium (Fig. 4A), which indicate that lovastatin-induced apoptosis was a consequence of inhibition of HMG-CoA reductase. We also examined the role of the cholesterol pathway in the apoptosis induced by lovastatin. As shown in Fig. 4A addition of squalene, an intermediate in the pathway leading to cholesterol synthesis did not prevent lovastatin-induced apoptosis. Similar results were obtained with mevastatin (Fig. 4B), a statin produced by the mould *Penicillium citrinium*. Mevastatin at doses of 5 and 20 μM induced apoptosis in MDA-MB231 cells and these effects were prevented by mevalonate added to the culture medium but not by cholesterol intermediate squalene.

The synthesis of cholesterol is a complex multi-step process [3]. Isoprenoids are important intermediates in the cholesterol biosynthetic pathway that serve as lipid attachments for a variety of signalling molecules, such as heterotrimeric G-proteins and the small GTP-binding proteins Ras and its related Ras-like proteins, such as Rho, Rac, Rab, Rap and Ral. Farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), two isoprenoids compounds produced in the cholesterol synthesis pathway, are bound to several cellular proteins, including small GTPases by a post-translational modification known as isoprenylation. This process involves the addition of a 15-carbon farnesyl chain in FPP (farnesylation) or a 20-carbon geranylgeranyl chain in GGPP (geranylgeranylation) to a cysteine sulfhydryl group near the carboxyl terminus. By inhibiting HMG-CoA reductase, statins can prevent the biosynthesis of isoprenoids such as FPP and GGPP. As a consequence, the diverse actions of the small GTP-binding proteins are reduced. To elucidate how statins were activating apoptosis in breast tumor cells, we examined the role of prenylation of proteins in the apoptosis induced by lovastatin and mevastatin in MDA-MB231 cells. We incubated the cells with the isoprenoids derivatives geranylgeranylpyrophosphate (GGPP) or farnesylpyrophosphate (FPP) and determined the sensitivity to statin-induced apoptosis. As shown in Fig. 4C, when cells were pretreated with GGPP or FPP and treated with lovastatin or mevastatin, statin-induced apoptosis was strongly reduced. Therefore, when protein prenylation is restored after statin treatment, apoptosis is not observed. These results indicate the importance of the inhibition of protein prenylation in the apoptotic cell death induced by statins in breast tumor cells. The translocation of Ras to the cell membrane is necessary for activity and is dependent on farnesylation. Similarly, attachment of Rho and Rac to the cell membrane is required for activity but, in contrast to Ras, these factors undergo ger-

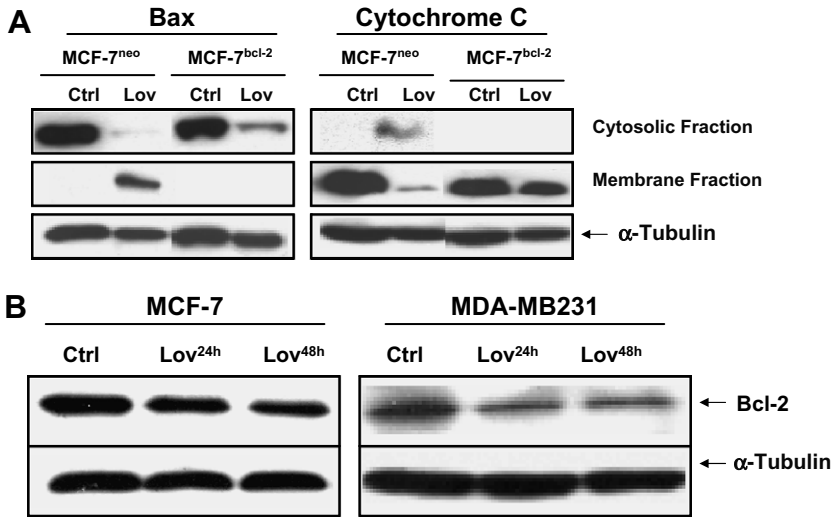

Fig. 3. Lovastatin induces the mitochondrial apoptotic pathway in breast tumor cells. (A) MCF-7 cells overexpressing Bcl-2 ($MCF7^{bcl-2}$) and their control cells ($MCF7^{neo}$) were seeded in 6-well plates (3×10^5 cells/well) and treated with lovastatin (100 μM in the presence of 10% serum) for 48 h. Cells were harvested and cytosolic and mitochondria-containing fractions were obtained as described in Section 2. Protein levels of Bax and cytochrome *c* were analyzed by Western blot. Similar results were obtained in two independent experiments. (B) 3×10^5 cells per well of MCF7 and MDA-MB231 cells were seeded in 6-well plates and treated with lovastatin (100 μM in the presence of 10% serum) for 24 h or 48 h. Total protein was harvested and levels of Bcl-2 were determined by Western blot analysis. α-Tubulin levels were used as loading controls. Similar results were obtained in two independent experiments.

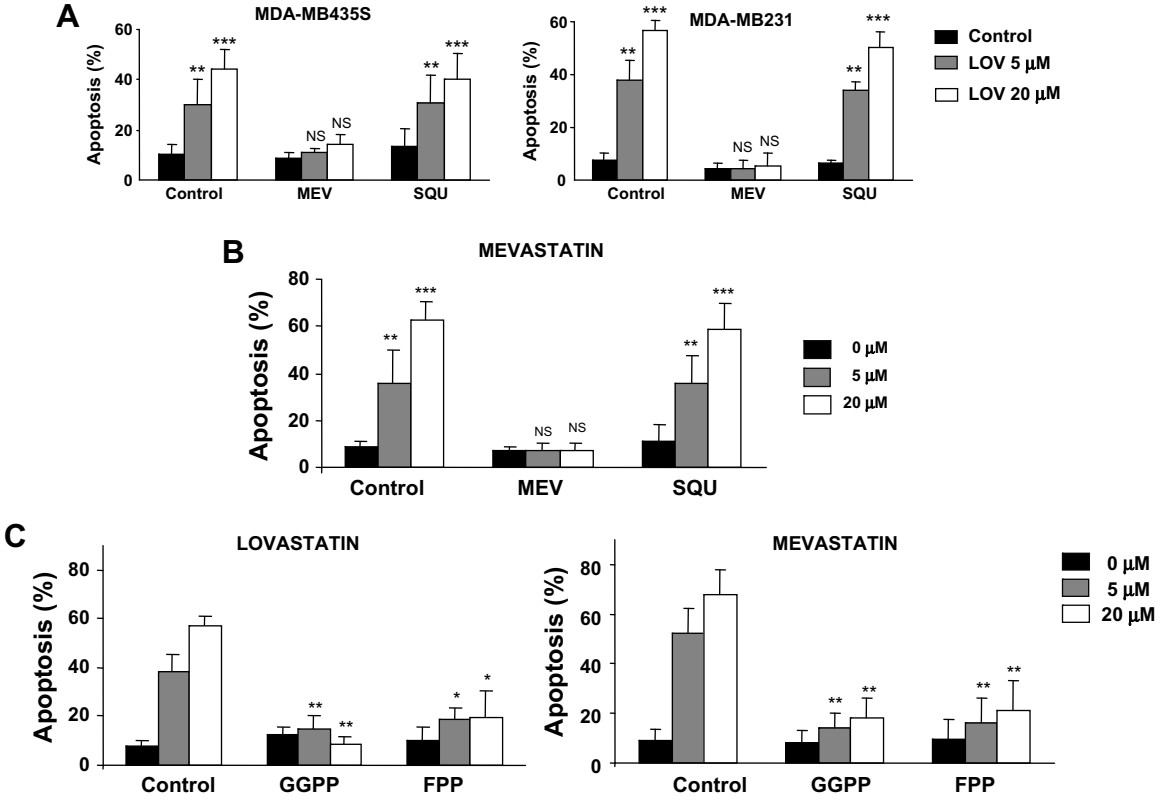

Fig. 4. Statins-induced apoptosis is mediated by HMG-CoA-Reductase inhibition and dependent on protein prenylation. MDA-MB435S (A) or MDA-MB231 (A and B) cells were seeded in 6-well plates (3×10^5 cells/well) and pretreated with mevalonic acid (MEV, 20 μM) or squalene (SQU, 20 μM) 1 h before the addition of lovastatin (A) or mevastatin (B) for 48 h in the presence of 0.5% foetal bovine serum. Apoptotic cell death was determined by flow cytometry as described in Section 2. Results show the means ± S.E. of three independent experiments. (C) MDA-MB231 cells were seeded as in (A) and pretreated with geranylgeranyl pyrophosphate (GGPP, 20 μM) or farnesyl pyrophosphate (FPP, 20 μM) 1 h before the addition of lovastatin or mevastatin for 48 h in the presence of 0.5% serum. Apoptotic cell death was determined as described in Section 2. Results show the means ± S.E. of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ as compared to untreated cells.

anylgeranylation. Our finding that restoration of either farnesylation or geranylgeranylation of proteins is sufficient to prevent statin-induced apoptosis in breast tumor cells suggest that different members of the small GTP-binding proteins family are involved in the mechanism of apoptosis induction by statins. An alternative explanation for these results is the unusual property of RhoB among prenylated proteins that can be farnesylated or geranylgeranylated [20]. Inhibition of small GTP-binding proteins prenylation by statins could affect various signalling pathways regulating cell survival [21]. In this respect, a role of Rho inhibition in the antitumor and proapoptotic effects of statins by inhibiting Rho-dependent NF-kB activation has been reported [19]. Abrogation of cell adhesion to the extracellular matrix has also been observed in cells treated with statins and this could affect cell survival [22].

We have observed that breast tumor cells overexpressing ErbB2 are markedly more resistant to the proapoptotic effects of statins than other breast tumor cell lines. Amplification or overexpression of ErbB2 in cancer cells confers resistance to apoptosis and promotes cell growth through Akt activation [23]. It has been recently shown that statins synergistically induce tumor cell death with inhibitors of the PI3K/Akt pathway [24]. Our results may indicate that in breast tumor cells expressing elevated levels of ErbB2 the constitutive activation of survival pathways like PI3K/Akt, MAPK/Erk1/2 and NF-kB may render these cells resistant to the proapoptotic effects of statins. In this context, statins could be valuable tools as antitumor agents in combination with pharmacological inhibitors of these survival pathways.

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